

Analysis of Subtelomeric Heterochromatin in the *Drosophila* Minichromosome *Dp1187* by Single *P* Element Insertional Mutagenesis

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ABSTRACT

We investigated whether single *P* element insertional mutagenesis could be used to analyze heterochromatin within the *Drosophila* minichromosome *Dp1187*. Forty-five insertions of the *P*[*lacZ,rosy*⁺] element onto *Dp1187* (recovered among 7,825 transpositions) were highly clustered. None was recovered in centromeric heterochromatin, but 39 occurred about 40 kb from the distal telomere within a 4.7-kb hotspot containing tandem copies of a novel 1.8-kb repetitive DNA sequence. The DNA within and distal to this region lacked essential genes and displayed several other properties characteristic of heterochromatin. The *rosy*⁺ genes within the inserted transposons were inhibited by position-effect variegation, and the subtelomeric region was underrepresented in polytene salivary gland cells. These experiments demonstrated that *P* elements preferentially transpose into a small subset of heterochromatic sites, providing a versatile method for studying the structure and function of these chromosome regions. This approach revealed that a *Drosophila* chromosome contains a large region of subtelomeric heterochromatin with specific structural and genetic properties.

CHROMOSOMES in multicellular eukaryotes have been studied extensively at the genetic and cytogenetic levels. However, much remains to be learned concerning the molecular structure of chromosomes and the mechanisms underlying chromosome behavior during the cell cycle and development. The study of small natural and artificial chromosomes has greatly aided the analysis of chromosome function in yeast (MURRAY and SZOSTAK 1983; NEWLON *et al.* 1991). For this reason we previously initiated molecular studies of a *Drosophila* minichromosome, *Dp(1;f)1187*, or *Dp1187* (KARPEN and SPRADLING 1990). *Dp1187* contains only 1000 kb of centromeric heterochromatin, and 300 kb of distal DNA, making it the smallest known functional chromosome in *Drosophila* and other multicellular eukaryotes. We now report the use of *P* element insertional mutagenesis to analyze the distal subtelomeric region of *Dp1187*.

Chromosomal telomeres carry out specialized functions essential for chromosome maintenance [reviewed by ZAKIAN (1989) and BLACKBURN (1991)]. The short GT-rich oligonucleotide repeats found at the extreme termini of chromosomes in a wide range of organisms serve as substrates for the enzyme telomerase (GREIDER and BLACKBURN 1985). The sequences added by telomerase counteract the 5' shortening of chromosomal molecules that would otherwise occur

due to the priming problem (WATSON 1972; CAVALLIER-SMITH 1974). In *Drosophila*, chromosomes bearing terminal deletions lose 50–75 bp of DNA per generation (BIESSMANN and MASON 1988; LEVIS 1989), presumably for this reason (LEVIS 1989; BIESSMANN, CARTER and MASON 1990). The structure of normal *Drosophila* telomeres has not been described, however, and the existence of terminal GT-rich repeats remains to be demonstrated.

Telomeric regions frequently contain other repeated DNA sequences in addition to hexanucleotide or oligonucleotide repeats. For example, most yeast chromosomes contain tandem repeats of a sequence called *Y'*, and an *X* sequence (CHAN and TYE 1983). Large arrays of repetitive sequences are found at the telomeres of many other organisms, including *Plasmodium* (PACE *et al.* 1987), *Secale* (BEDBROOK *et al.* 1980), *Chironomus* (SAIGA and EDSTRÖM 1985), and humans (COOKE, BROWN and RAPPOLD 1985; BROWN *et al.* 1990; DELANGE *et al.* 1990). A specific family of repeats called HeT has been mapped at many *Drosophila* telomeres (RUBIN 1978; YOUNG *et al.* 1983). The functions of these "subtelomeric" repeats are unclear, since they vary widely in amount between chromosomes and undergo rapid evolutionary changes (YOUNG *et al.* 1983; CORCORAN *et al.* 1988; ZAKIAN and BLANTON 1988). Recently, BIESSMANN *et al.* (1990) reported that HeT repeats can be added onto a broken chromosome end, possibly by retrotransposition. Sequence additions, along with telomere-telomere recombination, might contribute to the

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rapid changes observed in subtelomeric regions.

In *Drosophila*, telomeres have been associated with additional genetic and cytogenetic properties. Telomeres are generally thought to be heterochromatic (SCHULTZ 1947), and share common sequences with centric regions and the *Y* chromosome (YOUNG *et al.* 1983). It has been suggested by cytological examinations, but not proven by molecular analyses, that telomeric regions become underrepresented in polytene DNA (ROBERTS 1979). Rearrangements that juxtapose euchromatic and heterochromatic chromosome regions frequently cause genes near the breakpoint to display position-effect variegation (reviewed in SPOFFORD 1976; HENIKOFF 1990; SPRADLING and KARPEN 1990). The expression of a *white*⁺ gene relocated near the 3R telomere variegated (HAZELRIGG, LEVIS and RUBIN 1984; LEVIS, HAZELRIGG and RUBIN 1985), however, it remains unclear if telomeric regions are generally capable of causing variegated position effects.

Studies of *Dp1187* telomeres, and of its centromeric region, were limited by a lack of specific molecular probes and of mutations mapping to these regions. We therefore used insertional mutagenesis with single *P* elements (COOLEY, KELLEY and SPRADLING 1988) to determine if genetically marked *P* elements bearing unique sequence tags could be recovered in *Dp1187* heterochromatin. Heterochromatin is generally thought to be an infrequent target of *P* element insertion (ENGELS 1989), but our experiments revealed that a significant number of insertions occurred near the distal telomere of this minichromosome. These studies have provided new information concerning the structure and function of *Drosophila* telomeres, and their relationship to transposable elements.

MATERIALS AND METHODS

***Drosophila* stocks:** Flies were grown on standard corn meal/agar media [see ASHBURNER (1990)], at 22°. Unless stated otherwise, strains and mutations are as described in LINDSLEY and ZIMM (1992). The original stock containing *Dp1187* was provided by D. LINDSLEY, and was balanced with *XY^s·Y^t, Df(1)259 y w.* and *C(1)RA, l(1)J1 In(1)sc^e*. The *X* chromosome was found to have broken down, and the duplication was subsequently maintained in a *y* background, by selection for *y*⁺.

Transposon mutagenesis of *Dp1187*: Prior to initiating the screen, sublines of the *y ac PZ; cn; ry; Dp1187* stock, the *cn; ry Sb Δ2-3 ry⁺/TM3 Ubx* stock (see ROBERTSON and ENGELS 1989 for the derivation of the *Δ2-3* transposase-encoding transformant), and the *y; ry⁵⁰⁶* stock were started from single pair matings. Two sublines of each stock that showed very high levels of viability and fertility were used.

The crossing scheme used for transposon mutagenesis of *Dp1187* is diagrammed in Figure 1, and the results of the screen are summarized in Table 1. *F*₀ crosses were performed *en masse*, in bottles started with approximately 20 males and 20 females. Approximately 22,000 *F*₁ dysgenic males were individually mated in vials with two *y; ry⁵⁰⁶* virgin

females. Among an average of 100 *F*₂ progeny from each vial, *y⁺ ry⁺ Sb⁺* males were recognized as bearing new transpositions of *P[lacZ, rosy⁺]* (hereafter called *PZ*; see MŁODZIK and HIROMI 1992) not linked to the *X* chromosome. These males were mated individually with two *y; ry⁵⁰⁶* females, however, a maximum of only two *y⁺ ry⁺ Sb⁺* males were used from any one *F*₁ vial to avoid clusters, and males from the same vial were recorded and kept together for further study. Approximately 8,300 such *F*₂ crosses were established, including 950 pairs. In some *F*₁ crosses the transpositions all segregated from *Dp1187*, or segregated with the chromosome containing *Δ2-3*, hence the number of *F*₂ vials established was less than the total number of transpositions that occurred.

Lines containing insertions onto *Dp1187* were recognized by co-segregation of the *y⁺* and *ry⁺* markers in progeny from the *F*₂ outcrosses (at least some *y⁺ ry⁺* progeny, but no *y⁻ ry⁺*). Linkage of the *PZ* element to the *Y* chromosome was established by observing transmission of the *ry⁺* marker only from fathers to sons. Lines containing an insertion on *Dp1187* and a second insertion on another chromosome were recognized (despite the presence of *y⁻ ry⁺* animals) by observing that all *y⁺* progeny were *ry⁺*; the insertions were then separated in a subsequent generation. A total of 42 independent lines with insertions on *Dp1187* were eventually recovered (Table 2). Autosomal *PZ* insertions were balanced over *CyO*, *TM3 ry^{rk}*, or (rarely) *CxD* and recessive (or rare dominant) phenotypes associated with the insertion-bearing chromosome classified. A small number of lines with insertions on the 4th chromosome or lines that were not balanced over either *CyO* or *TM3* for undetermined reasons were discarded. In addition, ovaries were dissected from adults of each line, stained for β -galactosidase activity, and the expression pattern recorded. Subsequent molecular and phenotypic study revealed that approximately 50% of the lines isolated as pairs contained identical insertions, so the total number of independent insertions recovered was approximately 8,300 - 475 = 7,825. Some of these results will be presented in further detail elsewhere.

Plasmid rescue: Sequences flanking an insertion were rescued by first preparing DNA from ten adult males as described (BENDER, SPIERER and HOGNESS 1983). Following digestion with *Xba*I, or *Xba*I and *Spe*I, the reaction was diluted, ligated overnight at 15°, and used to transform competent *DH5* cells to kanamycin resistance, essentially as described (PIROTTA 1986; COOLEY, KELLEY and SPRADLING 1988). Approximately one colony was obtained for each fly equivalent of DNA used for transformation.

Restriction mapping using pulsed-field and conventional gel electrophoresis: The location of the *PZ* insertions in *Dp1187* was determined by pulsed-field Southern analysis, utilizing the *Not*I and *Sfi*I restriction map (see Figure 3) and the presence of sites for these enzymes within the *PZ* transposon. High molecular weight DNA was isolated from hand dissected larval brains and imaginal discs, or adult ovaries, using the agarose insert method described previously (KARPEN and SPRADLING 1990). DNA from each *Dp1187 PZ* strain was digested with *Not*I or *Sfi*I. Pulsed-field Southern blots (see KARPEN and SPRADLING 1990) were probed with a fragment (*pBS 12.1BH9*) corresponding to positions -80 to -89 on the *Dp1187* map (see Figure 3) for the *Not*I digests, and a fragment from -40 to -51.5 (*pBS TG1BP11.5*) for the *Sfi*I digests. The 1100-kb proximal *Not*I fragment was reduced in size by transposon insertions proximal to position -100 on the *Dp1187* map (see Figure 3), while insertions to the left of -40 reduced the size of the 250-kb distal *Sfi*I band. The orientation of the insertion then was determined by reprobing the blots with probes

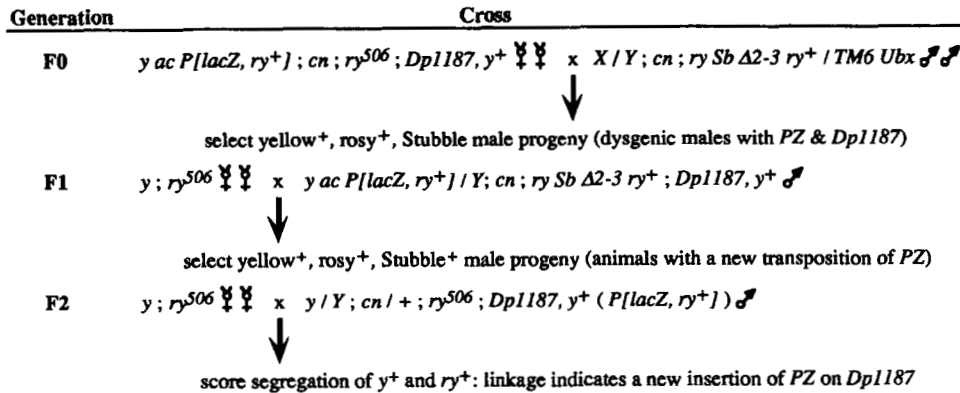


FIGURE 1.—Crossing scheme used for single PZ mutagenesis of Dp1187. See MATERIALS AND METHODS for details.

TABLE 1
Summary of PZ transposition screen

Class	Number
F ₁ crosses (approximate)	22,000
Transposition lines	8,300
Independent lines	7,825
Lethals: II + III	958
Female steriles: II + III	190
Male steriles: II + III	95
Insertions on Dp1187	42
Insertions on Y	24

Lines with recessive phenotypes represent those maintained in stock. A small fraction were lost during balancing. Approximately 200 lines bearing an identical third chromosome background female sterile mutation are not included among the steriles. Approximately 200 lethal lines that were accidentally contaminated are also not included in the lethal category.

homologous to the 5' (*pBS 5' Pend HR 0.55*) or 3' (*pBS 5' ryHR3.2*) ends of the transposon. Yeast chromosomes (strain YPH 149, supplied by PHIL HIETER), multimerized bacteriophage λ DNA, and a "5-kb ladder" (obtained from BioRad), served as size markers.

The map of the 9.9-kb *SpeI*-*XbaI* fragment was constructed using several sources of information. The genomic DNA present in the plasmid rescued from most of the 42 insertion lines was mapped using several restriction enzymes. To independently verify the location of each insertion, genomic DNAs (isolated from adult flies; BENDER, SPIERER and HOGNESS 1983) were subjected to conventional gel electrophoresis, and Southern blots were hybridized with the transposon-specific probes (see above), according to the methods described in KARPEN and SPRADLING 1990. The sizes of the *XbaI*, *XbaI*-*SpeI*, and *EcoRI* fragments were used to localize and orient the insertions within the distal hotspot (see Figure 5). When preliminary studies indicated that a strain contained a second insertion on an autosome (see Table 2), the insert-bearing duplication was isolated free of other *P* elements by outcrossing males to *y; ry⁵⁰⁶* females for at least two generations, then reanalyzed by Southern analysis.

DNA sequencing: DNA sequencing reactions were carried out essentially as described (LEVINE and SPRADLING 1985), using Sequenase 2.0 kits (U.S. Biochemical). Preparations of the marker rescued clones were used directly as templates, with a *P* element-specific primer located just inside the 5' inverted terminal repeat of the PZ element (sequence: 5' GTATACTTCGGTAAGCTTCGGCTATA 3'). To complete the sequence of the *SpeI*-*XbaI* fragment,

appropriate regions were subcloned and additional primers were synthesized. A summary of the information used to assemble the sequence is given in Figure 2. Sequence assembly and analysis was accomplished with the Genetics Computer Group software, version 7 (DEVEREAUX, HAEERLI and SMITHIES 1984).

In situ hybridization: *In situ* hybridization to polytene chromosomes was carried out as described previously (KARPEN and SPRADLING 1990). Briefly, RNA probes were prepared by transcribing plasmid DNAs (subcloned in Bluescript vectors) with T7 polymerase in the presence of [³⁵S] UTP and [³⁵S]CTP. Hybridization, washing and autoradiography were then performed as described.

DNA preparation and Southern blotting: DNA was prepared from brains, imaginal discs, and salivary glands of third instar larvae, as described previously (KARPEN and SPRADLING 1990). Electrophoresis, blotting, autoradiography, and quantitation of bands was as before (KARPEN and SPRADLING 1990).

RESULTS

Restriction mapping of Dp1187: Dp1187 is the smallest of a series of free X chromosome duplications (minichromosomes) generated by deleting all but the proximal and distal sequences within *In(1)sc⁸*, an X chromosome containing a large inversion [see KARPEN and SPRADLING (1990) for a description of the origin of Dp1187]. Previous studies using pulsed-field gel electrophoresis (KARPEN and SPRADLING 1990) defined the *sc⁸* breakpoint as 0 kb on the Dp1187 map, and showed that the chromosome contained approximately 1000 kb of centromeric heterochromatin proximal to this site, and about 300 kb of distal DNA (see Figure 3). Prior to initiating insertional mutagenesis, we carried out additional mapping to further define possible target sites. High molecular weight DNA was prepared from *Drosophila* tissues in agarose inserts, digested with restriction enzymes, and separated on pulsed-field gels containing size markers. Southern hybridization analysis revealed several facts about Dp1187 structure not reported previously. The size of the portion of Dp1187 corresponding to the normal X tip, *i.e.* sequences distal to the *sc⁸* breakpoint, measured 290 ± 5 kb (rather than 340 kb, as reported previously). A putative telomere was subsequently po-

TABLE 2
Properties of *Dp1187* insertions

Line	Site in <i>Dp1187</i> (kb) ^a	Site in hotspot (bp) ^b	Orientation ^c	Rosy expression ^d	Comments
0367	-249	4549/50	<	+++	
0401	-246	7305/6	>	+++	
0517	-246	ND	>	ND	Isolated in strain with second autosomal insert
0801	-248	4936/7	<	ND	Isolated in strain with second autosomal insert
0809	-246	6742/3	>	++	
1401	-247	5860/1	>	++	
1601	-247	ND	>	+++	
1630	-247	6406/7	<	++	
1801	-247	6397/8	>	++	
1803	-242		<	ND	Isolated in strain with second autosomal insert; isolated as part of a premeiotic cluster
2001	-249	4549/50	<	wt	2 inserts on <i>Dp1187</i>
	-85		<		
2202	-247	ND	<	ND	Isolated in strain with second autosomal insert
2622	-250	3567/8	<	++	
2801	-246	ND	>	ND	Isolated in strain with second autosomal insert
3001	-246	6628/9	>	+++	
3401	-251	2575/6	<	++	
3402	-250	3198/9	<	+++	
3601	-250	ND	<	++	Terminal deficiency
3901	-249	3706/7	>	++	
4001	-246	ND	>	wt	2 inserts in <i>Dp1187</i> hotspot
4003	-246	7305/6	>	++	Isolated in strain with second autosomal insert
4201	-246	6569/70	<	+++	
4401	-246	7339/40	>	wt	
4404	-249	ND	<	+++	
5402	-249	ND	<	ND	Isolated in strain with second autosomal insert
5403	-248	5301/2	>	+++	Isolated as part of a premeiotic cluster
6201	-246	6751/2	>	+++	
6231	-75		<	wt	
6401	-248	ND	>	+++	
6601	+900		ND	+	Unstable centromeric insert (see text)
7201	ND	ND	ND	ND	2 inserts in <i>Dp1187</i> hotspot
7401	-248	5451/2	<	+++	
7403	-247	ND	<	wt	
7606	-249	4540/1	>	+++	
8002	-75		<	wt	
8601	-250	3006/7	<	+++	
8602	-247	6397/8	>	+++	
8801	-80		<	wt	
9211	-247	6397/8	>	ND	Isolated in strain with second autosomal insert
9901	-248	4713/4	>	+++	
10240	-246	ND	>	ND	Isolated in strain with second autosomal insert
10500	-246	7305/6	<	ND	Isolated in strain with second autosomal insert

^a Approximate position of *PZ* insertions; kb = kilobases from the *sc⁸* breakpoint (=0 kb). See Figures 3 and 5. ND = not determined.

^b Position of the *PZ* insertion in the *Dp1187* hotspot; bp = basepairs proximal to the *SpeI* site (see Figure 7).

^c Orientation of the *PZ* element within *Dp1187*. Arrowhead indicates the 5' *P-lacZ* end of the *PZ* element; the *Dp1187* centromere is to the right, the euchromatic telomere to the left (see Figure 5).

^d Distinguishing weak *ry⁺* from *ry⁻* is difficult. Lines were therefore categorized according to the following scale, based on counts of >200 flies per line: wt = 0–5% of the progeny *ry⁻*, +++ = 6–25% *ry⁻*, ++ = 26–49% *ry⁻*, + = 50–100% *ry⁻*. Note that the presence of a second insert affected the classification of some lines (i.e., compare 0367 and 2001). wt, wild type.

sitioned at coordinate -290 by more precise methods (see below).

In addition, detailed mapping of the first 200 kb of the centric heterochromatin revealed evidence of substructure. Southern blots of the gels were probed with a 3.7-kb *XhoI-HindIII* fragment adjacent to the *sc⁸* breakpoint (pBSscXR3.7) (KARPEN and SPRADLING 1990), to more accurately map the location of the first

site for each enzyme within the centromeric heterochromatin. The first 50 kb contained few restriction sites (Figure 3), as expected for a region of simple sequence DNA. The 359 bp (or 1.688) "complex" satellite DNA (HSIEH and BRUTLAG 1979) was suspected to lie at the *sc⁸* breakpoint from cytological analyses (HILLIKER and APPELS 1982); this has been confirmed by cloning and sequencing a restriction

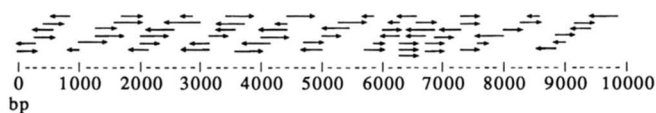


FIGURE 2.—Overview of the DNA sequencing strategy used for the *SpeI*-*XbaI* fragment. Arrows indicate the location, orientation and size of sequenced fragments used to assemble the 9,872-bp sequence shown in Figure 5. bp = base pairs, where 0 refers to the distal-most (*SpeI*), and 10000 the centromere-proximal (*XbaI*), ends of the fragment.

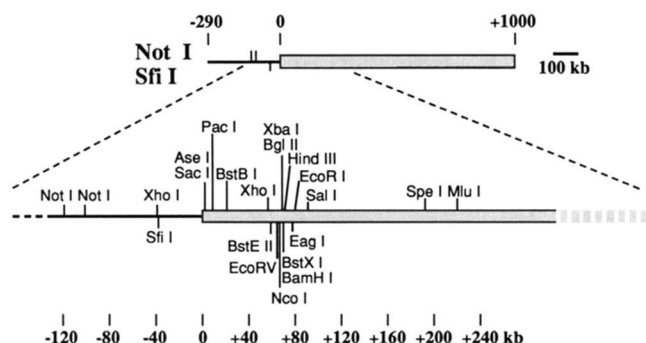


FIGURE 3.—Molecular structure of *Dp1187*. The restriction map of *Dp1187* is shown above, with sites for the rare-cutting enzymes *NotI* and *SfiI* indicated by hashmarks. Values indicate distance in kilobases from the *sc*⁸ breakpoint (= 0) into the euchromatin (solid line, - values) and the heterochromatin (stippled bar, + values). Detailed restriction map of the region surrounding the *sc*⁸ breakpoint is shown below. Only the first restriction site proximal to the breakpoint is shown for the enzymes that digest the heterochromatin. For clarity, only the *NotI*, *SfiI* and *XhoI* sites are shown in the euchromatin.

fragment that spans the *Dp1187* breakpoint (R. GLASER, G. KARPEN and A. SPRADLING, unpublished). The two enzymes that cut close to the *sc*⁸ breakpoint, *AseI* and *SacI* (Figure 3), are known to have sites within the 359-bp sequence (HSIEH and BRUTLAG 1979). Of the remaining enzymes tested, 12/15 first cut between +55 and +90 suggesting that approximately 50 kb of simple sequence DNA is followed by an "island" of more complex DNA between +55 and +90. The presence of complex DNA within the centromeric region encouraged the view that *P* element insertions might occur within them.

A limited amount of data suggested that simple sequence DNA might alternate with "islands" of more complex sequences throughout *Drosophila* centromeric heterochromatin. When the pulse-field gel Southern blots used in these mapping experiments were rehybridized with satellite DNA-specific probes (359-bp complex satellite, 1.672 = AATAT and 1.705 = AAGAG, LOHE and BRUTLAG 1986) a heterogeneous pattern of bands between 50–900 kb was observed (data not shown). Most *Drosophila* satellite DNAs are based on simple penta- or heptanucleotide repeats and would not be expected to contain sites for most of the enzymes tested (LOHE and BRUTLAG 1986). The observed sizes were therefore difficult to reconcile with a model in which simple sequence DNA stretches

uninterrupted across the 15–20-Mb centromere regions of the major chromosomes. Two enzymes, *SpeI* and *MluI*, did not cut within *Dp1187* until positions +180 and +215. This may indicate the presence of another region of simple sequence DNA followed by a second island.

Insertional mutagenesis of *Dp1187* heterochromatin; a new approach to genomic structural analysis: Genomic regions rich in repetitive DNA sequences are difficult to analyze by standard cloning methods. Approximately 120 kb of DNA distal to the *sc*⁸ breakpoint was cloned from standard genomic libraries by chromosome walking (FLEMING, DESIMONE and WHITE 1989; KARPEN and SPRADLING 1990). However, attempts to walk more distally (and within the centromeric region) were discouraged when regions of repeated DNAs began to be encountered. In principle, insertional mutagenesis with single *P* elements (COOLEY, KELLEY and SPRADLING 1988) could solve many of the problems associated with studying large chromosome segments lacking unique sequences. The presence of genetically marked insertions would greatly facilitate structural analysis, since each insertion would contain unique DNA sequences that could be used for restriction mapping. Furthermore, the chromosome region could be mutagenized by imprecisely excising inserted transposons, facilitating subsequent functional studies.

P element insertional mutagenesis, however, has generally been thought to be of little use in analyzing heterochromatic sequences. *P* elements located within heterochromatic regions have only rarely been observed in natural populations, or following transformation (ENGELS 1989). The paucity of characterized insertions into heterochromatin could be caused by an inability of *P* elements to transpose into these regions, due to a lack of favorable target sites or because of the chromatin environment. Alternatively, heterochromatic *P* insertions might be unable to express their marker genes and become underrepresented in polytene cells, making either genetic or physical detection difficult. A few *P* element insertions within heterochromatin of the 4th and Y chromosomes have been recovered previously, and lines bearing these insertions expressed the marker genes present on the transposon at reduced levels (SPRADLING and RUBIN 1983; LEVIS 1989; BERG and SPRADLING 1991).

We therefore designed an experiment to determine if insertional mutagenesis could be used to analyze *Dp1187* heterochromatin. A large mutagenesis screen was carried out in which a single *PZ* element (MLODZIK and HIROMI 1992), located initially on the X chromosome, was mobilized in a strain that also carried *Dp1187* (see MATERIALS AND METHODS). The *PZ* element was selected as the insertional mutagen, since it

carries an enhancer-sensitive *P-lacZ* fusion gene for enhancer trapping, as well as sequences allowing marker rescue in *Escherichia coli*. Furthermore, we preferred using the *rosy*⁺ (*ry*⁺) gene as a marker because very low levels of *ry*⁺ function are required to produce wild-type eye pigmentation. From a total of 8300 lines containing new insertions (representing approximately 7825 independent insertions), 42 lines were recovered that contained independent *PZ* insertions on *Dp1187* (Table 1). Subsequent molecular analyses revealed that a total of 45 insertions were actually present in these lines (Table 2). This frequency ($45/7825 = 0.58\%$) was similar to that expected assuming all *Dp1187* genomic DNA was available for insertion in the F1 males ($1.3 \text{ Mb}/180 \text{ Mb} = 0.72\%$), but higher than expected if only euchromatin was a target ($0.29 \text{ Mb}/107 \text{ Mb} = 0.27\%$).

The location of the *PZ* insertion in each strain was determined initially by mapping new *NotI* and *SfiI* restriction sites caused by the presence of the *PZ* element in their resident minichromosomes (Figure 4). Subsequently, genomic DNA flanking the 5' end of each insertion was recovered by marker rescue, and mapped in greater detail with restriction enzymes. Flanking DNAs cloned by marker rescue were compared to the known genomic structure determined from Southern blots, to minimize the possibility that clones had undergone rearrangements during isolation and growth in *E. coli*.

The distribution of insertions along the *Dp1187* map was extremely nonuniform (Figure 5). A major "hotspot" located near the distal telomere, 246–251 kb from the *sc*⁸ breakpoint (coordinate –246 to –251), contained 39 of the 45 insertions. However, within this 4.7-kb region, insertions occurred at many different sites and in both orientations (Table 2). The insertion in strain 1803 mapped a few kilobases proximal to the hotspot, while four other insertions were positioned between –75 and –85, a well studied region of euchromatin. Only one line, 6601, contained a candidate insertion within *Dp1187*'s 1000-kb segment of centromeric heterochromatin. Initially, this line expressed *ry*⁺ very weakly, but showed clear genetic linkage to *y*⁺ for several generations. However, the *ry*⁺ marker subsequently became linked to chromosome 3. Mapping with *NotI* revealed a structural change within *Dp1187* at +900 but no evidence of *P* element sequences (data not shown). We have been unable to determine whether *PZ* initially inserted onto *Dp1187* in strain 6601 and then subsequently was transferred to chromosome 3 by a heterochromatic exchange event, or if an original insertion on chromosome 3 was associated with unstable rearrangements that caused *ry*⁺ and *y*⁺ to appear linked.

The subtelomeric region distal to the hotspot: The presence of insertions near the distal *Dp1187* telomere

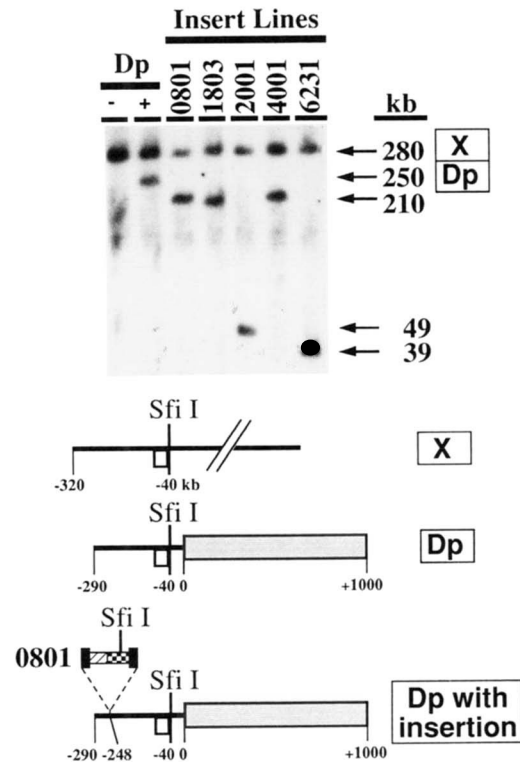


FIGURE 4.—Mapping new *PZ* insertions on *Dp1187* by pulsed-field gel electrophoresis. Pulsed-field Southern hybridization analysis of *SfiI*-digested ovary DNA from *Dp1187* insertion lines was performed as described in MATERIALS AND METHODS, and a representative autoradiogram is shown. The genotypes of the lines are *y*; *ry*⁵⁰⁶ (= *Dp*–), *y*; *ry*⁵⁰⁶; *Dp(1;f)1187*, *y*⁺ (= *Dp*+), and *y*; *ry*⁵⁰⁶; *Dp* (with *PZ* insertion), *y*⁺ *ry*⁺ (= 0801–6231). Sizes of restriction fragments are indicated to the right; X = X chromosome-specific fragment, *Dp* = *Dp1187*-specific fragment. Diagrams of the structure of this region of the X, *Dp1187* and *Dp0801* chromosomes are shown below, with the locations of the *SfiI* sites indicated. Open box denotes the probe used in this experiment (*TG1BP11.5*). *NotI* digests, and probe *12.1BH9*, were used to analyze the structure of the derivatives, proximal to –100, in a similar manner, capitalizing on the presence of two *NotI* sites in the *PZ* element. For a description of other symbols refer to the Figure 3 legend. See Figure 5 and legend for the structure of the *PZ* element.

allowed the subtelomeric region to be mapped in greater detail. Southern blots with genomic DNA flanking the hotspot insertions revealed only the presence of repetitive DNA sequences (see below). Consequently, we carried out additional mapping using line 3401, in which the single copy sequences within the *PZ* transposon were located closest to the telomere (Figure 6A). These studies verified that both the *NotI* and *SfiI* distal "sites" mapped to the same location. Furthermore, two other enzymes, *XbaI* and *XhoI*, also appeared to cleave at this same position, while no enzyme was found with a more distal site. These observations suggested that the minichromosome distal telomere resides at this location. The existence of a telomere at –290 is consistent with size measurements of uncut *Dp1187*, that suggested a total length of about 1300 kb (data not shown).

The insertion in strain 3601 differed from the

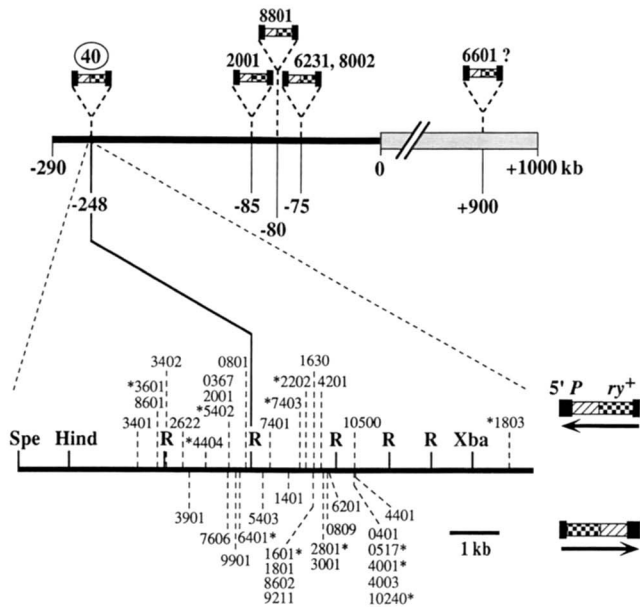


FIGURE 5.—Location of PZ insertions on *Dp1187*. The approximate location and orientation of the PZ insertions, determined by pulsed-field Southern analysis (see MATERIALS AND METHODS and Figure 4), is shown above. The locations and orientations of 37 of the 40 insertions within the subtelomeric hotspot, determined by conventional Southern analysis and/or analysis of plasmid rescued flanking DNA, are displayed below. Numbers indicate strain name. Arrow points toward the 5' *P* end of the PZ transposon. Black boxes = *P* ends; box with diagonal lines = 5' *P*-*lacZ* fusion plus the *kanamycin^R* gene and the bacterial origin of replication; box with checkerboard is the *rosy⁺* gene (*ry⁺*). Spe = *SpeI*, Hind = *HindIII*, R = *EcoRI*, Xba = *XbaI*. See Table 2 for nucleotide locations within the *SpeI*-*XbaI* fragment. Refer to the Figure 3 legend for all other symbols.

others in the region distal to the hotspot. 3601 contained an insertion at -250, and in addition, the chromosome appeared to be broken ~650 bp distal from the 5' *P* end. A series of restriction enzymes showed the existence of apparent sites at this position (Figure 6B), although such sites are absent in the other lines (*i.e.*, *Dp6201*, Figure 6B). Furthermore, the small terminal bands migrated heterogeneously on gels only in 3601 (Figure 6B, *EcoRI* digest), as previously observed for broken chromosome ends (BIESSMANN and MASON 1988; LEVIS 1989). Perhaps the insertion event that produced the 3601 strain simultaneously caused a deletion of the distal 40 kb. Alternatively, a secondary *P* element-catalyzed event may have caused the deletion during germ-line cell divisions subsequent to insertion, similar to the breakage events observed by LEVIS (1989) following experimental mobilization of a subterminal *P* insertion.

We used the terminally deleted *Dp1187* derivative in strain 3601 (*Dp3601*) to learn if any genes reside in the terminal 40 kb that lies distal to the hotspot. Appropriate crosses were made to a series of mutated or deleted X chromosomes, including *Df(1)sc⁸* which lacks all the DNA sequences distal to the *sc⁸* breakpoint (Figure 6C). *Dp3601* rescued the lethality asso-

ciated with the genes uncovered by these deficiencies, and restored the flies to a fully wild-type state. We conclude that all the essential genes on the X, and on *Dp1187*, lie proximal to -250, and that the terminal 40 kb of the minichromosome lacks genes with easily detectable phenotypes. Whether redundant functions are encoded by repetitive sequences within this region could not be addressed by these studies.

DNA sequence of the hotspot region: The wide distribution of PZ insertions throughout the 4.7-kb hotspot greatly facilitated determination of its DNA sequence. A primer specific for the 5' *P* end was used to sequence flanking genomic DNA within most of the rescued plasmid templates, revealing much of the sequence of both strands. The sequences were then extended using oligonucleotides homologous to the ends of the initial runs, and templates from subclones of the marker-rescued plasmids (see MATERIALS AND METHODS). Since the insertion sites were unambiguously positioned within the hotspot based on the genomic and plasmid maps, it was possible to join the sequences into a continuous 10-kb segment spanning the entire *SpeI*-*XbaI* fragment (Figure 7). Furthermore, this method revealed the exact nucleotide at which most insertions occurred (Table 2).

The hotspot region contained a series of different repetitive elements arranged in an organized, specific pattern. Starting at the distal *SpeI* site (Figure 7), the first 2163 bp comprised a member of the HeT family (RUBIN 1978; YOUNG *et al.* 1983; TRAVERSE and PARDUE 1989; BIESSMANN *et al.* 1990), followed by 3.1 nearly perfect tandem repeats of a novel 1.8-kb sequence (called 1.8A-1.8D), then 2.2 tandem repeats of a 0.9-kb sequence (called 0.9A and 0.9B). At the junction between the different types of repeated elements, the 5'-most element in each group was truncated or internally deleted. These large repeats in turn contain internally repeated, short sequence elements (~80-170 bp).

The HeT element strongly resembled HeT repeats sequenced previously (VALGEIRSDÓTTIR, TRAVERSE and PARDUE 1990), except a gap was present between bases 910 and 1120, and the first 450 bases of the HeT DNA was present as a tandem, diverged repeat. Heterogeneity among members of this family has been noted previously (TRAVERSE and PARDUE 1989). The 3' terminus of the HeT element joined the first 1.8-kb tandem repeat in a region of five A residues. This junction was identical in structure to that recently observed at the tip of two terminally deficient chromosomes that had been "capped" by addition of an HeT element (BIESSMANN *et al.* 1990); both the presence of poly(A) residues and the position of the junction within the HeT sequence were the same in the *Dp1187* hotspot and in the terminal additions. The 5 A's may have been part of an HeT mRNA poly(A)

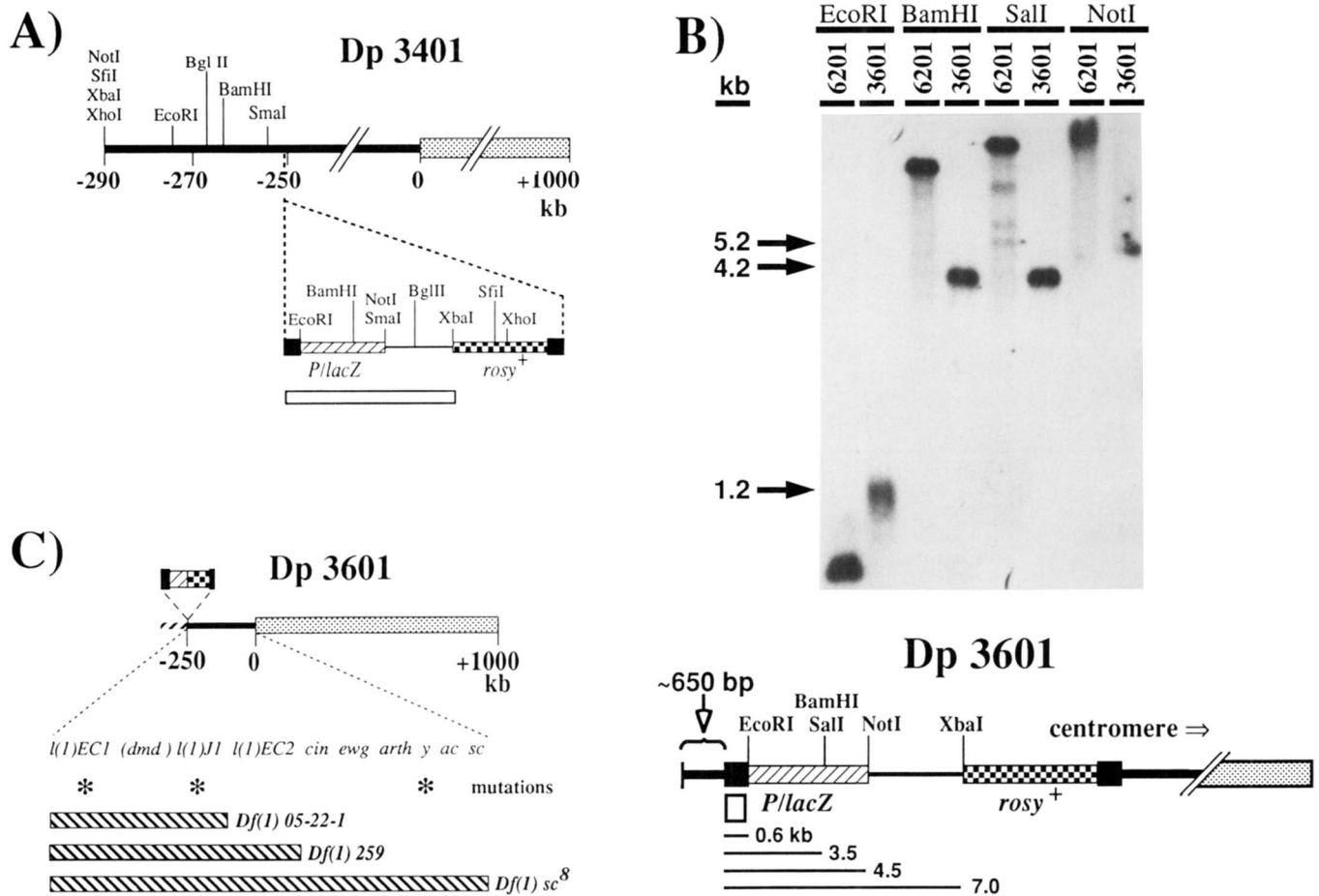


FIGURE 6.—Structure of the subtelomeric region of *Dp1187* elucidated by analyses of *PZ* insertion lines. A) The restriction map distal to the insertional hotspot was determined by pulsed-field Southern analysis, using the indicated enzymes, DNA from line *Dp3401* (the distal-most insert in the *Dp1187* hotspot), and a single-copy probe from within the transposon (open box). B) *Dp3601* contains a terminal deficiency. The restriction map of the region distal to two different hotspot insertions, in lines *Dp3601* and *Dp6201*, was determined by conventional electrophoresis and Southern analysis. The restriction map for *Dp3601* is shown below the autoradiogram; probe = the 5' *P* end (open box). The coincidence of sites for all 6 enzymes ~650 bp distal to the end of the 3601 insertion (the *XbaI* digest yielded a fragment of 7.6 kb, data not shown), plus the heterogeneous migration of the short *EcoRI* fragments, indicate that 3601 is terminally deleted. C) Complementation of *X* chromosome mutations (asterisks) and deletions (bars with diagonal lines indicate the genes that are absent in the deficiencies) by *Dp3601* localizes the distal-most *X*-linked genes to the region proximal to the 3601 insertion. Other symbols as in previous figures.

tail prior to its reverse transcription and ligation to the distal 1.8-kb repeat at some previous time in the evolution of the *Dp1187* telomeric region.

The two complete 1.8-kb repeats (1.8B = 1871 bp and 1.8C = 1857 bp, Figure 7) are >95% homologous over positions 2372–4243 and 4244–6101. These sequences exhibit no significant homology to anything in the available databases, and have no large open reading frames. Each 1.8-kb repeat itself contains three tandem copies of a 173/173/161-bp subrepeat (dashed line, hollow arrow in Figure 7); the two 5' most repeats contain a 12-bp insertion (box with slashes) absent in the third repeat. The 1.8-kb repeats each contain single copies of 80-bp (double line) and 160-bp (solid line and arrow) elements. The first copy of the 1.8-kb repeat (1.8A) is only 205 bp in length; it is truncated at its 5' end (the junction with HeT) in comparison to 1.8B and 1.8C, but is >99% homolo-

gous from the truncated 160 bp repeat through to the end of the 80-bp element. Finally, the fourth repeat (1.8D = 1574 bp, positions 6102–7676) is >95% homologous to 1.8B and C, except that ~280 bp are deleted near the 3' end of 1.8D, including the entire 160-bp repeat.

The *SpeI*-*XbaI* fragment ends with two repeats (0.9A = 874 bp, positions 7809–8683, 0.9B = 910 bp, positions 8685–9595) that also have no homology to current database entries, and lack significant open reading frames. These elements each contain two copies of a 167-bp repeat (jagged line, half-arrow, Figure 7), plus one complete and one truncated copy of a 160-bp element that is identical to that present in the 1.8-kb repeats (solid line, solid arrow Figure 7; see above). The truncated 160-bp repeats are joined at their 3' ends to one of the complete 167-bp repeats. The region between 1.8D and 0.9A is in fact a deleted

version of a 0.9-kb repeat, containing a nearly complete 167-bp repeat fused to a truncated 160-bp element. Thus, the region proximal to the 1.8A–D repeats, from 7677 to 9595, contains a series of related sequences whose distal-most element is rearranged in comparison to the more proximal elements. Finally, the last 276 bp of the hotspot region (9596–9872) contains sequences that are unrelated to the more distal elements. Further analysis will be necessary to determine if these sequences are repeated in the region proximal to the *Xba*I site, and thus follow the pattern established for the organization of the HeT, 1.8- and 0.9-kb elements.

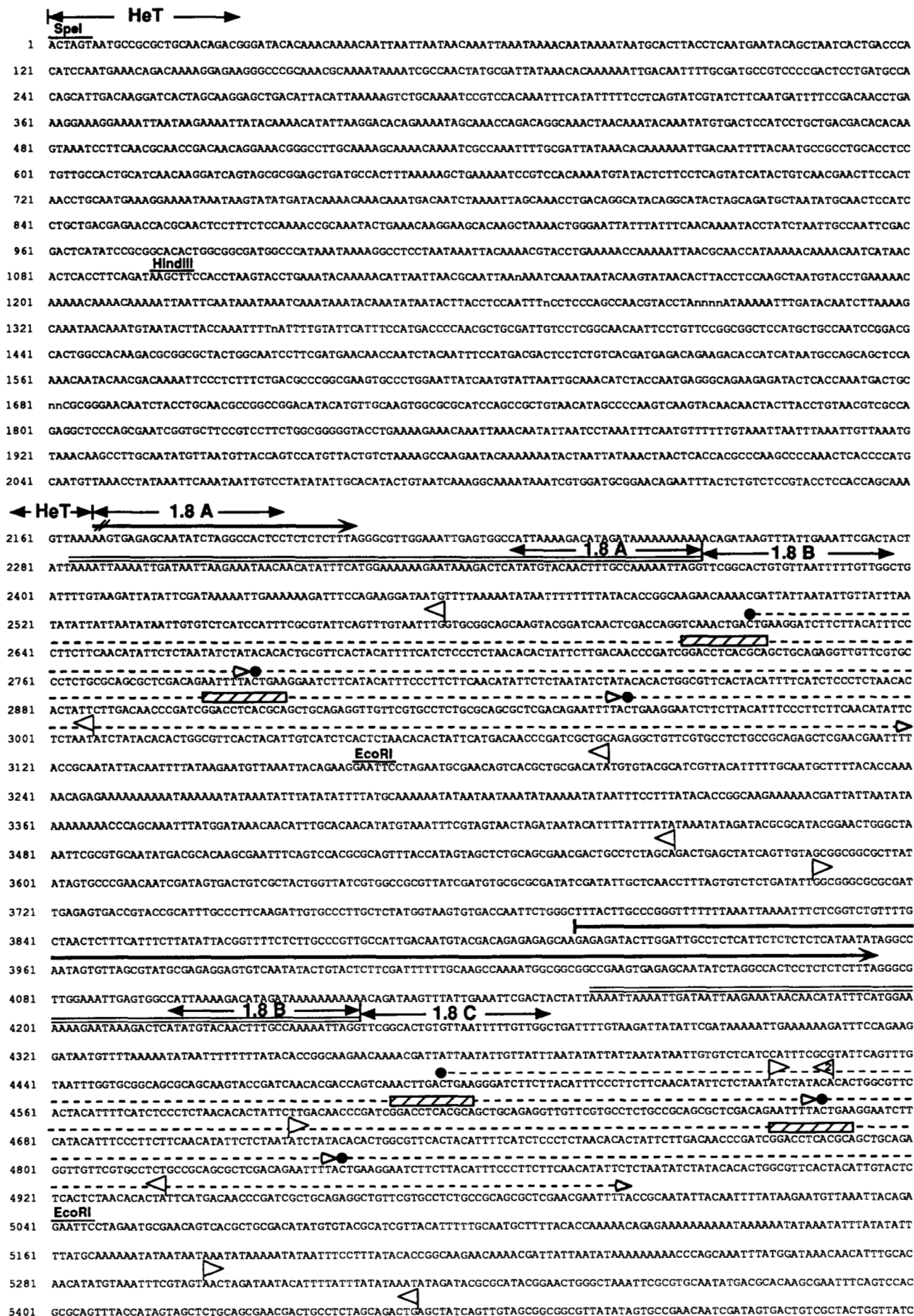
Distribution of *P* element insertion sites within the hotspot: The *P* element insertions within the hotspot region were distributed nonrandomly, yet displayed a regional, rather than a site-specific, insertional preference. *PZ* elements only inserted within a 4.7-kb portion of the 1.8-kb repeats; no elements were recovered in the distal HeT element or the proximal DNA (see Figure 5, and Figure 7 flags). The only exception was the insertion in line 1803, which mapped by restriction analysis to be less than 1 kb proximal to the *Xba*I site. The sequences at the 5' end of this insertion have not been determined, and could turn out to match sequences within the *Spe*I-*Xba*I fragment. There was a disparity in the distribution of insertions among the different 1.8-kb elements, despite their similarity in sequence. Surprisingly, the internally deleted 1.8D repeat received more insertions than the complete elements 1.8B and C (Figures 5 and 7; 7 *PZ* insertions in 1.8B, 11 in 1.8C, and 18 in 1.8D).

Insertion sites were distributed throughout the individual 1.8-kb repeats, and were rarely located at the same nucleotide (only 3 clusters of 2, 3 and 3 insertions, Figure 7). However, there was some preference for the 173/161-bp subrepeats, since 56% of the insertions (14/25 sequenced insertion sites) occurred there, while these elements constitute only 27% of the DNA in the 1.8-kb element. Furthermore, a compilation of insertion sites for all of the 173/161-bp repeats revealed that most (12/14) of the insertions inserted within an 11-bp region of the repeat (Figure 8A). Two sites, at the 5' and 3' ends of this region, contained multiple insertions (8 and 4 insertions, respectively). Compiling data from the different 1.8-kb repeats demonstrated that four independent *PZ* insertions also occurred within another specific sequence located outside the 173/161-bp repeats (Figure 8B). The flanking sequences at all three sites with multiple insertions are significantly diverged from each other, and from the *P* insertion 8-bp consensus sequence derived by O'HARE and RUBIN (1983). However, the region 3' to one of these clusters (CACTGGCG; Figure 8A, stippled box) contains a 7/8 match to the *P*

element insertional hotspot in the *singed* locus (CACTGGAG; ROIHA, RUBIN and O'HARE 1988; HAWLEY *et al.* 1988). We conclude that the 1.8-kb repeats displayed the properties of a regional hotspot for *P* element insertion, with some sequence specificity in site choice within the region. The molecular mechanisms responsible for the unusual distribution of *PZ* insertions in this hotspot have yet to be elucidated (see DISCUSSION).

Multiple copies of the 1.8-kb hotspot sequence are located predominantly at salivary gland telomeres: A 1.8-kb *Eco*RI fragment containing a single 1.8-kb repeat and a 0.9-kb *Eco*RI fragment containing one of the proximal repeats were subcloned from the plasmid rescued clones. Southern analysis of whole *Drosophila* genomic DNA indicated that sequences within the 1.8-kb element are moderately repeated within the *Drosophila melanogaster* genome (50–100 copies, data not shown). These sequences are present predominantly as 0.95 kb and 0.85 kb repeats. The location of these sequences was determined by *in situ* hybridization to salivary gland polytene chromosomes. Both fragments strongly labeled the telomere regions of two chromosomes, 2R and 3R (Figure 9, large arrows). The chromocenter region also was labeled weakly (Figure 9A, bracket), as was one basal euchromatic site (Figure 9A, small arrow). Strong labeling of the X chromosome tip was not observed, presumably because the X chromosome in the $y; ry^{506}$ strain used to prepare chromosomes contained at most a few copies of these sequences. The labeling at the chromosome tips frequently was observed to extend along ectopic fibers that emanate from the tips and extend to other chromosome regions (Figure 9, open arrow). In some cases, labeling completely spanned the ectopic fiber linking the 2R and 3R tips, which are rich in these sequences (Figure 9B, open arrow).

Insertions in the hotspot region are subject to position effect: The subtelomeric region of *Dp1187* exhibited a deleterious position effect on the expression of the inserted ry^+ gene. Unlike the majority of *PZ* insertions, including those in the *Dp1187* euchromatin, all lines containing a single insertion in the hotspot produced progeny with weak and variable expression of *ry*. A fraction of the progeny were phenotypically ry^- ; this number depended on the site of insertion (Table 2). Among the 8300 lines recovered in the screen, the weak and variable *ry* phenotypes associated with hotspot insertions were similar only to those displayed by 24 insertions linked to the Y chromosome (data not shown). Regardless of the eye phenotype, all progeny transmitted the minichromosome and expressed the *yellow*⁺ (y^+) gene it contained. Loss of ry^+ expression was not due to germinal mutation, since progeny of ry^- individuals showed the same range of phenotypes as their ry^+ sibs. The re-



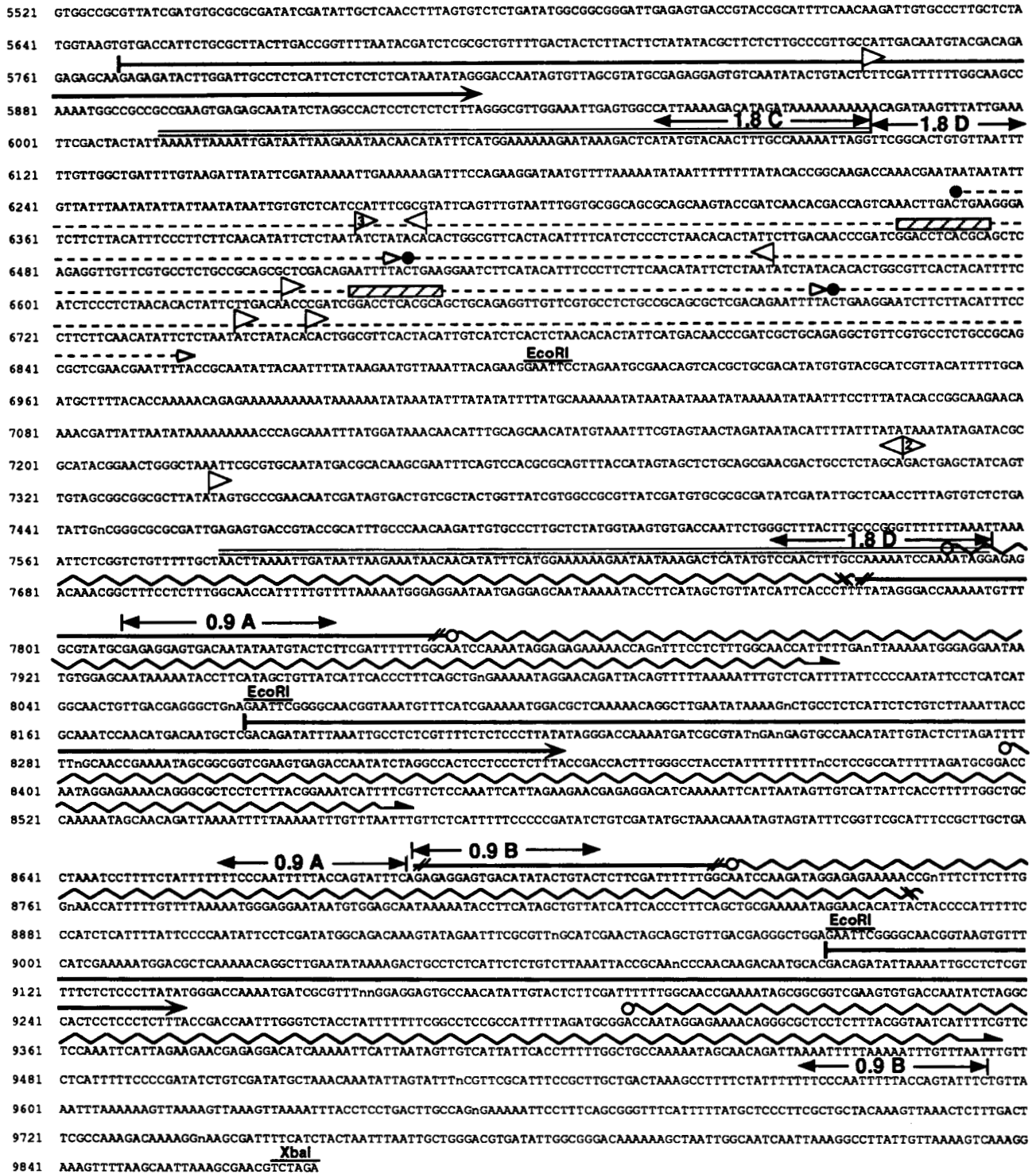


FIGURE 7.—DNA sequence and structural features of the *SpeI*-*XbaI* hotspot region. The 9872 bp DNA sequence starts with the sequences closest to the distal telomere (the *SpeI* site, positions 1–6; see Figure 5 for the position of this region within *Dp1187*), and ends with the centromere-proximal *XbaI* site (positions 9867–9872). Structural features of this region are noted above each line. These include restriction sites (*SpeI*, *HindIII*, *EcoRI* and *XbaI*) and major repeats (HeT, 1.8A–D; 0.9A and B). Subrepeats located within the major repeats are designated by double lines (80-bp repeat), dashed lines/open arrowheads (173/173/161-bp tandem repeats), solid lines/solid arrowheads (160-bp repeat) and jagged lines/half arrowheads (167-bp repeat). Boxes with diagonal lines are 12-bp insertions that constitute the only difference between the 173- and 161-bp repeats. // indicates subrepeats that are truncated at their 5' and/or 3' ends. Flags indicate the positions of the sequenced *PZ* insertions, and the flag direction denotes the transposon's orientation (tip = 5' P end; see Figure 5). 1803 is inserted 3' to the *XbaI* site, and is not included in this sequence. Numbers inside flags represent the number of independent insertions at that site, if greater than one. See Figure 5 and Table 2 for insertion line designations.

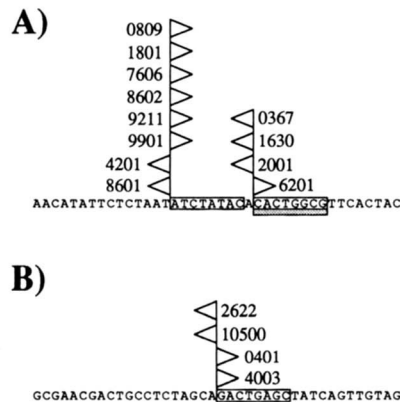


FIGURE 8.—Sequence-specific PZ insertion within the 1.8-kb repeats. Homologous subrepeats from the 1.8-kb elements were aligned to identify sequence-specific insertional hotspots not apparent from the dispersion of PZ insertions throughout 1.8B, C and D. Only three sequences, which correspond to the three clusters noted in Figure 7, were found to contain more than one insertion when summed in this manner; two (A) were located 9 bp apart in the 173/161-bp repeats and one (B) in the 3' portion of the 1.8-kb repeat. Dp line numbers are next to flags that indicate the orientation of each insertion (see Figures 5 and 7). Boxed sequences indicate the 8-bp genomic DNA duplicated upon P element insertion (O'HARE and RUBIN 1983). Stippled box highlights a 7/8-bp match to a P element insertion hotspot identified at the *singed* locus (ROIHA, RUBIN and O'HARE 1988; HAWLEY *et al.* 1988).

ductions were due to position effects, since transposons remobilized to other locations subsequently expressed *ry*⁺ in a normal, stable manner (J. TOWER, G. KARPEN, N. CRAIG and A. SPRADLING, unpublished).

Genes juxtaposed with heterochromatic regions frequently display variegated position effects [reviewed by SPOFFORD (1976), HENIKOFF (1990) and SPRADLING and KARPEN (1990)]. We tested whether *ry*⁺ expression from hotspot insertions was affected by the dosage of the Y chromosome, a strong modifier of position-effect variegation. All the hotspot insertion lines tested failed to express *ry*⁺ in X/O males, but expressed fully wild-type eye color in X⁺Y/Y males. Thus, the position effect on *ry*⁺ expression responded as expected for a variegated position effect. Previously, the R401.1 insertion within repetitive sequences on the 4th chromosome was shown to exhibit variegated *ry*⁺ expression with similar characteristics (SPRADLING and RUBIN 1983; DANIELS *et al.* 1986; A. SPRADLING and D. THOMPSON, unpublished).

The *Dp1187* telomere becomes underrepresented in the DNA of polytene cells: Centromeric heterochromatin is severely underrepresented in chromosomes of many polytene tissues (reviewed by SPRADLING and ORR-WEAVER 1987). Cytological studies have led to suggestions that telomeric regions also may be underrepresented (ROBERTS 1979). To determine if the relative copy number of the *Dp1187* telomere decreased as a result of salivary gland polytenization, we compared larval salivary gland DNA to DNA from predominantly diploid imaginal disc tissue. Sub-

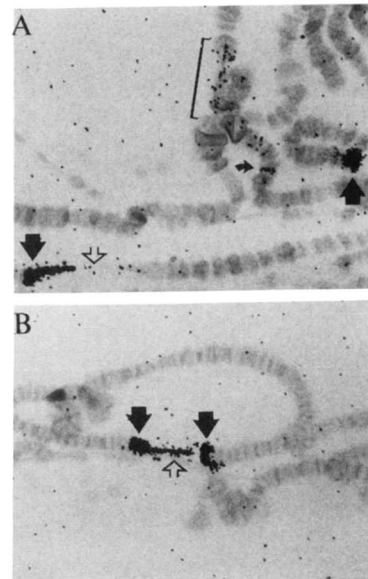


FIGURE 9.—*In situ* hybridization of 1.8-kb repeat sequences to Canton-S salivary gland polytene chromosomes. The distribution of 1.8-kb element sequences within the genome was determined to be primarily telomeric (2R and 3R, large solid arrows) and centromeric heterochromatin (chromocenter, bracketed area). One basal euchromatic site was observed (small arrow). Ectopic fibers joining chromosomal telomeres (open arrows) were frequently labeled. Sometimes (A) only one of the two tips contained significant amounts of the 1.8-kb sequences, whereas in other cases (B) the 1.8-kb element was well represented on both tips (2R and 3R).

cloned, repeated DNAs from the hotspot region could not be used as probes in these experiments, since it was difficult to recognize the bands specific for the duplication among the many complementary fragments on a Southern blot. This is a general problem encountered when analyzing the copy number of subtelomeric repeats, since all characterized to date in *Drosophila* are present in both centromeric and subtelomeric regions, and cannot be distinguished by restriction fragment polymorphisms. However, unique sequences within transposons inserted in the *Dp1187* hotspot provided a tag to specifically follow the behavior of the minichromosome telomere.

DNA from *Dp3401* males with X/O and X/Y sex chromosome constitutions was examined to determine if telomeric sequences are underrepresented in polytene nuclei, and if DNA copy number in telomeric regions is sensitive to a strong modifier of position-effect variegation. High molecular weight DNA isolated from late third instar larval salivary glands and imaginal discs was digested with *Bgl*II or *Xba*I, separated on a pulsed-field gel, and hybridized with a *lacZ* probe specific for the insertion (Figure 10). The 48-kb *Xba*I fragment that extended all the way from the hotspot region to the telomere was strongly underrepresented in X/O male salivary gland polytenized DNA, compared to the disc diploid DNA (13-fold, Table 3). The amount of underrepresentation of the *Xba*I fragment was reduced to 2.6-fold when a Y chromosome

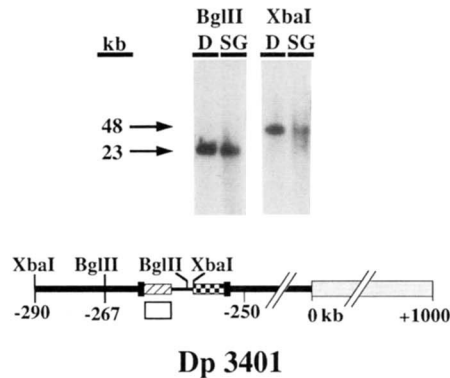


FIGURE 10.—Underrepresentation of the subtelomeric region in salivary gland DNA. Pulsed-field Southern hybridization analysis of *y/Y; ry⁵⁰⁶; Dp3401, y⁺ ry⁺* larval imaginal disc (D) diploid cells and salivary gland (SG) polytene nuclei was performed as described in MATERIALS AND METHODS. The structure of the subtelomeric region in this line is shown below. Symbols are the same as in previous figures. The autoradiogram shown was produced by probing with *lacZ* sequences (open box). Reprobing the same blots with *ry⁺* sequences (5' *ryHR3.2*) showed that equivalent amounts of DNA were loaded in the D and SG lanes for each digest (data not shown). Note that the underrepresentation of the terminal *XbaI* fragment in polytene DNA is greater than for the subterminal *BglIII* fragment (see Table 3).

was present. However, the 23-kb *BglIII* fragment containing sequences from the transposon insertion to position -267 was underrepresented only 1.6-fold in salivary gland DNA compared to diploid DNA. Both restriction fragments migrated more heterogeneously when prepared from salivary gland DNA than diploid DNA, possibly as a consequence of an elimination mechanism that was proposed to be responsible for sequence underrepresentation (KARPEN and SPRADLING 1990; R. GLASER, G. KARPEN and A. SPRADLING, unpublished). The reduced intensity and heterogeneous migration were not simply the result of nonspecific DNA degradation, since restriction fragments of similar or greater size that lay within euchromatin (the endogenous *rosy* locus) were unaffected.

DISCUSSION

A *P* element hotspot within subtelomeric, repetitive DNA: Our results demonstrated that *P* element insertions compatible with marker gene expression occurred selectively but frequently within repetitive, subtelomeric DNA. The fraction of insertions into the *Dp1187* hotspot, 39/7,825 (0.5%), was several fold greater than any other hotspot observed in our screen, such as lethal *stripe* insertions (19/7,825). The insertion frequency into the *XbaI-SpeI* fragment appears similar to the average frequency inferred for individual elements from data on previously described hotspots such as *singed* (ENGELS 1989). The recovery of insertions at more than one site within a hotspot was reported previously for insertions at *singed* (ROIHA, RUBIN and O'HARE 1988; HAWLEY *et al.* 1988) and

TABLE 3

Underrepresentation of *Dp1187* subtelomeric DNA in salivary gland polytene nuclei

Genotype ↓	Fold-underrepresented ^a	
	<i>BglIII</i> fragment ^b	<i>XbaI</i> fragment ^b
<i>X,y/O;Dp 3401,y⁺</i>	ND	13
<i>X,y/Y;Dp 3401,y⁺</i>	1.6	2.6

^a Values indicate the amount of underrepresentation of *Dp1187* subtelomeric DNA in polytene salivary gland nuclei (fold difference in the amount of DNA), when compared to the same region in the predominantly diploid imaginal discs (see MATERIALS AND METHODS, and Figure 10). The values include normalization for differences in the total amount of DNA loaded in the lanes, determined by reprobing with a *ry⁺* probe and measuring the representation of the endogenous *ry⁺* fragment (see MATERIALS AND METHODS).

^b See Figures 6A and 10 for the size and position of the restriction fragments. ND = not determined.

Notch (KELLEY *et al.* 1987). In these cases, however, insertions were spread over only a few hundred base pairs, significantly smaller than the 4.7-kb hotspot near the *Dp1187* telomere. Some structural or functional property of the hotspot region, for example transcriptional activity or chromatin structure in male germ-line cells, might explain the generally high frequency of insertion, and the apparent preference for the proximal 1.8 kb repeat. However, some role for sequence specificity in site choice within regional hotspots is suggested by the striking homology between one of the *Dp1187* multiple insertion sites and the *singed* hotspot (ROIHA, RUBIN and O'HARE 1988; HAWLEY *et al.* 1988).

The screen employed would have detected any insertion on *Dp1187* that was still capable of expressing the *ry⁺* gene, but none were recovered in centromeric heterochromatin or other subtelomeric regions. The simple sequences that constitute much of centromeric heterochromatin might lack *P* element target sequences. However, the identification of an island of complex sequences within *Dp1187* heterochromatin makes it more likely that potential *P* target sequences reside in the centromeric heterochromatin. Since all the hotspot insertions tested suffered strong position effects on *ry⁺*, and similar reductions were seen with insertions on the *Y* chromosome (BERG and SPRADLING 1991; G. KARPEN and A. SPRADLING unpublished), 4th chromosome (SPRADLING and RUBIN 1983), and near the 3R telomere (HAZELRIGG, LEVIS and RUBIN 1984; LEVIS, HAZELRIGG and RUBIN 1985), marker gene expression is likely to be a limiting factor. Our results suggest that this problem can be mitigated by scoring insertions in the presence of an extra *Y* chromosome, or in a genotype containing suppressors of variegation.

Our choice of the *Dp1187* minichromosome and insertional mutagenesis greatly assisted the subsequent structural analysis of the repetitive, subtelomeric region. Direct cloning of repetitive DNA into

plasmids may have helped avoid sequence rearrangements. Equally valuable was the ability to map each insertion site in the genomic DNA using pulsed field and standard gel electrophoresis. It would have been much more difficult to correctly position the transposon insertion sites, and to delineate the structure of each 1.8 kb repeat, without this information. The introduced single-copy sequences within the transposon allowed the copy number of the tip region to be studied in polytene cells. Furthermore, the DNA in this region can now be manipulated genetically by remobilizing the insertions (J. TOWER, G. KARPEN, N. CRAIG and A. SPRADLING, unpublished). Many of these advantages would hold for *P* insertions within other heterochromatic sites.

Structure and evolution of subtelomeric heterochromatin: The subtelomeric region of *Dp1187* that we analyzed displayed several properties characteristic of heterochromatin. First, sequences from this region were repetitive, but were found primarily at telomeres and in centromeric regions. Second, no known or essential genes were present in the 40-kb region distal to the 3601 insertion. Third, the euchromatic *ry⁺* gene suffered variegated position effects upon insertion within the *Dp1187* subtelomeric region. Finally, the copy number of restriction fragments from the subtelomeric region was underrepresented in DNA from polytene salivary gland cells, relative to euchromatic fragments. All these properties are shared by centromeric heterochromatin in *Drosophila* chromosomes.

Sequence analysis of the 10-kb subtelomeric *XbaI-SpeI* fragment revealed a high degree of sequence structure. Distal HeT sequences were joined to tandem repeats of the 1.8-kb element, followed by imperfect tandem repeats of the 0.9-kb sequence. HeT elements are located near other *Drosophila* telomeres (RUBIN 1978; YOUNG *et al.* 1983), and some of these chromosome tips also contain 1.8-kb repeats (AJIOKA 1987). A *P* element insertion in the subtelomeric region of chromosome 3R (HAZELRIGG, LEVIS and RUBIN 1984) has recently been shown to reside in a sequence related to the 1.8-kb element (R. LEVIS, personal communication). Further analyses of normal and newly generated telomeres will be needed to determine if the organization of HeT, 1.8 kb and possibly other repeated sequences is conserved in *Drosophila*, like the *X* and *Y'* elements of yeast.

The addition and loss of DNA sequences may be a regular feature of subtelomeric chromosome regions. BIESSMANN *et al.* (1990) recently demonstrated that HeT elements can be added onto terminally deleted chromosome ends. R. LEVIS, R. GANESAN, K. HOUTCHENS and F. SHEEN (in preparation) have proposed that sequence additions to normal chromosome termini by retrotransposition might counterbalance

the small loss of DNA due to the priming problem. The absence of a strict mechanism to balance the rate of addition and loss would provide an explanation for both the existence and evolutionary instability of telomeric heterochromatin. The structure of the *SpeI-XbaI* fragment provided some support for this model. The HeT element was linked with the first 1.8-kb repeat via a short stretch of A residues, like the HeT addition events observed by BIESSMANN *et al.* (1990). Possibly, at some previous time the telomere of the *X* chromosome progenitor of *Dp1187* terminated in 1.8-kb repeats, when this HeT was added. Subsequently, the equilibrium may have favored addition events so that 40 kb of additional sequences accumulated. Sequencing of DNA lying distal to the *SpeI* site would test some of the predictions of this model.

The frequent insertion of a transposable element into repetitive target sequences, as observed in our studies, provides an additional mechanism that could contribute to the maintenance and evolution of telomeres, as well as other heterochromatic regions not susceptible to terminal additions. The acquisition of 1.8-kb elements in a subtelomeric region might be followed closely by the accumulation of *P* elements. If transposons themselves served as targets for other transposable elements, once started this process would lead to the rapid growth in the size and complexity of this region. The structure of the junctions between members of the 1.8- and 0.9-kb repeats do not provide sufficient information to support or discount this model. Further studies of the structure and behavior of these elements are needed to resolve these questions. However, some other mechanism(s) may have generated the tandem repetition of major (*i.e.*, 1.8A-D) and minor (*i.e.*, 173/173/161-bp) repeats. Perhaps the poorly understood mechanism that is involved in maintaining tandem repetitions within other heterochromatic regions, including satellite DNA and ribosomal DNA repeats, also acts on these sequences.

Sequence underrepresentation of telomeric heterochromatin: Our experiments provided the first direct demonstration that subtelomeric sequences become underrepresented in a *Drosophila* polytene cell type, the salivary gland. The existence of copy number changes in telomere regions has previously been postulated on cytological grounds (ROBERTS 1979). Both heterochromatic sequence underrepresentation and copy number decreases within "constrictions" in polytene chromosomes have invariably been assumed to result from differential DNA replication (reviewed in SPRADLING and ORR-WEAVER 1987). However, recent studies of *Dp1187* led us to propose that a process of DNA elimination similar to that observed in some nematodes and in ciliate macronuclei was actually responsible for the decreases in heterochromatic sequences in polyploid and polytene cells (KARPEN and

SPRADLING 1990; SPRADLING and KARPEN 1990; SPRADLING *et al.* 1992). Telomeres of nonhomologous polytene chromosomes display a strong tendency to be joined together by thin strands termed "ectopic fibers" (HINTON and ATWOOD 1941). The labeling along ectopic fibers spanning the two 1.8-kb-rich telomeres that was observed in polytene chromosomes can readily be explained by the elimination model. Religation of individual DNA strands following elimination of more distal heterochromatin at both tips could join strands from separate chromosomes. Stretching of such covalently joined chromatids during chromosome preparation would produce ectopic strands rich in the 1.8-kb repeats. Other subtelomeric heterochromatic sequences, such as HeT repeats, also would be expected to become located in some ectopic fibers (depending on the site of breakage and ligation), and labeling of ectopic fibers with HeT probes has been observed previously (RUBIN 1978; YOUNG *et al.* 1983). Another puzzling attribute of polytene chromosomes, the occasional linkage of telomeric and chromocentral regions via ectopic fibers, also can be explained if the elimination of sequences in both heterochromatic regions is occasionally followed by centromere-telomere, rather than telomere-telomere or centromere-centromere, religation.

Underrepresentation of DNA in telomeric regions can also explain the cytological appearance of polytene chromosome tips. The morphologies of X chromosome tips are altered significantly by the addition or removal of Y chromosomes (SCHULTZ 1947). These morphological differences may be caused by alterations in telomeric sequence representation, which we observed to be influenced in *trans* by the number of Y chromosomes. In addition, the morphology of telomeres has been observed to vary among salivary gland cells from the same larva (DOBZHANSKY 1944; LEFEVRE 1976; ROBERTS 1979). Recent studies show that the level of heterochromatin-associated underrepresentation differs among polytene nuclei (KARPEN and SPRADLING 1990); thus, variations in tip morphology could result from cell-to-cell differences in the underrepresentation process.

Sequence underrepresentation was observed in the subtelomeric region of *Dp1187* DNA isolated from salivary glands; a terminal 48-kb fragment was underrepresented more than a subterminal 27-kb fragment. A copy number gradient in salivary gland DNA was found previously within the first 103 kb of *Dp1187* euchromatin that resides adjacent to centromeric heterochromatin (KARPEN and SPRADLING 1990). Genes within both regions were subject to variegated position effects. We argued previously that the reductions in DNA copy number might be sufficient to explain the reduction in y^+ expression in variegating chromosomes (KARPEN and SPRADLING 1990). However,

in the case of the hotspot insertions, the ry^+ gene within *PZ* was reduced less than twofold, while ry^+ expression must have been severely reduced, perhaps as much as 100-fold, to explain the effect on eye color (CHOVNIK, GELBART and MCCARRON 1977). Consequently, the heterochromatic position effects on these subtelomeric insertions predominantly suppress gene expression, rather than DNA copy number.

Hotspot insertion and *P* element regulation: Previous studies revealed that some wild strains of *D. melanogaster* frequently carry *P* element inserts in region 1A (AJIOKA and EANES 1989). The chromosomal location of these insertions are likely to lie within the hotspot sequence we analyzed, since several were mapped to a "1.9"-kb *EcoRI* repeat that hybridized to the tips of 2R and 3R (AJIOKA 1987). The identity of these sites remains to be directly verified. Nonetheless, the presence of an insertion hotspot in our experiments differs from the observed lack of elevated *P* insertion into region 1A in a chromosome tested by these authors (AJIOKA and EANES 1989; also see RONSSERAY, LEHMANN and ANXOLABEHRE 1991). The existence of such a hotspot may vary between strains. The fact that 1.9-kb repeats were not observed on all X chromosome tips examined might provide the basis for these differences (AJIOKA 1987).

Recent studies suggested that *P* insertions within the 1A hotspot might exhibit an unusual ability to repress *P* element movement. Intact *P* elements produce a 66-kD protein that can repress *P* element activity (MISRA and RIO 1990), but only low levels of repression that varied depending on the insertion site were associated with any single element, even if it had been engineered to produce large amounts of 66-kD protein [reviewed by RIO (1990)]. A strain derived from the wild in which all the *P* elements were removed except those located at position 1A appeared to be an exception (RONSSERAY, LEHMANN and ANXOLABEHRE 1991). Although containing only two complete *P* elements, it strongly repressed germ-line *P* element activity, and repression was maternally transmitted to progeny (RONSSERAY, LEHMANN and ANXOLABEHRE 1991). *P* insertions within the hotspot may be subject to position effects that enhance their effectiveness as regulators of transposition.

The preferential insertion of *P* elements into a heterochromatic site where they may play a role in regulating *P* element activity has potential implications for the evolutionary role of transposable elements. Previously, it has been argued that *Drosophila* transposons are unlikely to confer a selective advantage because individual sites detectable by *in situ* hybridization are usually occupied only at low frequency within a population (see AJIOKA and HARTL 1989). However, many transposable element family members are located in centromeric heterochromatin, where

they frequently occupy conserved sites (BUCHETON *et al.* 1984; SHEVELYOV, BALAKIREVA and GVOZDEV 1989). Transposons may be able to efficiently integrate into certain heterochromatic regions, where they can effectively regulate the activity and copy number of other element copies, an ability that is likely to be of selective value.

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